

UNITED STATES ENVIRONMENTAL PROTECTION AGENCY
WASHINGTON, D.C. 20460

JAN 16 1996

MEMORANDUM

OFFICE OF
PREVENTION, PESTICIDES AND
TOXIC SUBSTANCES

SUBJECT: Review of AgrEvo's Data on the Safety of Phosphinothricin Acetyltransferase (PAT) for Use in Corn and Soybeans

TO: Michael Mendelsohn
Regulatory Action Leader
Biopesticides & Pollution Prevention Division
(7501W)

FROM: John L. Kough, Ph.D., Biologist
Biopesticides & Pollution Prevention Division
(7501W)

THROUGH: Roy D. Sjoblad, Ph.D., Senior Scientist *K*
Biopesticides & Pollution Prevention Division
(7501W)

DATA REVIEW RECORD

Active Ingredients: Glufosinate-ammonium
Product Name: Liberty Herbicide
ID No: 045639-ROO
Submission No: S493377
Chemical No: 128850
DP Barcode: D219909
MRID: 437669-28 through 33 and 437784-03

ACTION REQUESTED

To review the data from AgrEvo relating to the safety of the phosphinothricin acetyltransferase gene and enzyme present in corn and soybean plants rendering these plants resistant to glufosinate-ammonium herbicides.

BACKGROUND

Resistance to certain herbicides has recently been introduced into the genome of some crop plants. The presence of an enzyme such as phosphinothricin acetyltransferase (PAT) in these plants allows them to tolerate the application of the broad spectrum glufosinate-ammonium herbicide. As a part of the application to broaden the use designation for the glufosinate-ammonium herbicide, the company has generated data to demonstrate the safety of the introduced gene and expressed protein. This is necessary as this protein has never been a part of the food supply or expressed in a food plant.

BPPD RECOMMENDATIONS

There are currently no guidelines in place for the studies as submitted. The data presented here includes: two key toxicology studies that are scientifically acceptable (MRID 437669-33 & 437784-03), one acceptable product characterization study (MRID 437669-32), three studies that have certain deficiencies but which supplement the conclusions of the acceptable studies (MRID 437669-28, 437669-29 & 437669-31) and one study that does not add to the toxicological evaluation (MRID 437669-30). While no acute oral toxicity test was performed, these data are adequate to address the rapid degradation of the PAT enzyme and its relation to other PAT type enzymatic proteins. An exemption from the requirement for a food tolerance for the presence of the PAT enzyme itself in all raw agricultural commodities can be supported with this data. This review does not address the safety of phosphothricin or its metabolites in the presence of the PAT enzyme.

SUMMARY OF REVIEWS

MRID 437669-28: The phosphinothricin acetyl transferase (PAT) enzyme appears to acetylate only phosphinothricin and is not affected by the presence of the 21 amino acids tested. There is no indication PAT is able to acetylate these other amino acids. PAT, either purified or in a plant extract, is rapidly degraded in the presence of full strength canine gastric fluid. This PAT degradation occurs at a slower rate under higher pH incubation or when the PAT is presented as a plant extract. The presence of the PAT enzyme in a transgenic tomato did not apparently affect the levels of the inherent plant toxicant tomatine in any sampled tissue compared to a wildtype control.

CLASSIFICATION: Supplemental. The data summaries are not acceptable without the methods used to generate the numbers are presented in detail. Based on other information presented in this package, this study does not need to be upgraded.

MRID 437669-29: The incubation of a crude plant extract with detectable PAT activity in porcine and bovine stomach fluids caused a rapid decrease in the detectable level of PAT activity. If the pH of these fluids was raised, the PAT activity did not decrease as rapidly.

CLASSIFICATION: Supplemental. Some details of the analysis were not provided such as source and method of obtaining the digestive fluids, PAT assay incubation times and determination of PAT specific activity. Based on other information presented in this package, this study does not need to be upgraded.

MRID 437669-30: The PAT gene was not detectable by PCR after an hour's incubation of HCN92 crude plant extracts incubated in normal gastric fluid from cow and chicken. Functional copies of the PAT gene were detectable by PCR in all HCN92 crude plant extracts treated with gastric fluids with increased pH. Purified DNA from HCN92 was not able to transform *Escherichia coli* cells before or after gastric fluid incubation.

CLASSIFICATION: Supplemental.

MRID 437669-31: PAT enzyme activity was found in crude extracts of corn roots, leaves, stems and seed for two different transformation events. Pollen did not have detectable PAT activity.

CLASSIFICATION: Supplemental. No PAT values for control non-transformed tissue extracts were presented.

MRID 437669-32: PAT protein was detectable by western blot in the root and leaf extract of two transformed lines of corn (T14 & T25) and a line of canola (HCN92). No PAT was visible in the wild-type corn or canola.

CLASSIFICATION: Acceptable.

MRID 437669-33: The DNA and amino acid sequence data indicate that the PAT protein is not related to proteins known to be toxic or allergenic to mammalian species. The PAT proteins appear to be most closely related to themselves and are not apparently closely related to other acetyl transferase enzymes.

CLASSIFICATION: Acceptable.

MRID 437784-03: PAT enzyme, either purified or in a plant extract, is rapidly degraded in gastric fluids as monitored by immunorecognition and enzyme activity. If pepsin is omitted from the gastric fluid, the PAT enzyme is fairly stable being recognized in a western blot after 180 minutes and is still enzymatically active (~10% full activity) after 120 minutes.

CLASSIFICATION: Acceptable.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD JK
Secondary Reviewer: Roy D. Sjoblad, Ph.D., Microbiologist, BPPD RD

STUDY TYPE: Product Chemistry
MRID NO: 437669-28
CHEMICAL NO: 128850 Glufosinate-ammonium
TEST MATERIAL: L-Phosphinothricin N-Acetyltransferase
STUDY NO: A50188
SPONSOR: AgrEvo USA Company, Wilmington, DE
TESTING FACILITY: Hoechst Schering, Frankfurt, Federal republic of Germany
TITLE OF REPORT: L-Phosphinothricin N-Acetyltransferase Biochemical Characterization
AUTHOR: Dr. Arno Schulz
STUDY COMPLETED: May 13, 1993
CONCLUSION: The phosphinothricin acetyl transferase (PAT) enzyme appears to acetylate only phosphinothricin and is not affected by the presence of the 21 amino acids tested. There is no indication PAT is able to acetylate these other amino acids. PAT, either purified or in a plant extract, is rapidly degraded in the presence of full strength canine gastric fluid. This PAT degradation occurs at a slower rate under higher pH incubation or when the PAT is presented as a plant extract. The presence of the PAT enzyme in a transgenic tomato did not apparently affect the levels of the inherent plant toxicant tomatine in any sampled tissue compared to a wildtype control.
CLASSIFICATION: Supplemental. The data summaries are not acceptable without the methods used to generate the numbers are presented in detail. Based on other information presented in this package, this study does not need to be upgraded.

STUDY DESIGN

The L-Phosphinothricin N-Acetyltransferase (PAT) enzyme was characterized using the standard biochemical methods of substrate specificity, substrate and enzyme concentration, pH, temperature and molecular weight. In addition the enzyme's stability to canine gastric fluid and its possible effect on the production of inherent toxicants such as tomatine n tomato were examined.

STUDY METHODS

Purification

The exact purification methods used were not described. However, it was stated that the PAT was purified to homogeneity using ammonium sulfate precipitation, Sepharose-Q anion chromatography, gel filtration with Ultrogel AcA44 and chromatofocussing with PBE94.

pH and Temperature Optima

The enzyme was characterized for these two parameters but the methods are not given.

Enzyme Kinetics

The dependency of enzymatic activity on the concentration of substrate and enzyme itself was examined although no description of the methods employed was given. The concentration of the invariant component was not given although this can be assumed to be in excess if this was a classic K_m determination.

Enzyme Substrate Specificity

The specificity of the PAT enzyme for its substrate phosphinothricin was examined using ^{14}C phosphinothricin and acetyl-CoA with sufficient PAT enzyme to yield a 50% conversion ($\frac{1}{2} K_{\text{max}}$?) of phosphinothricin to acetyl-phosphinothricin. This reaction was then compared in the presence of potential competing substrates, notably the 19 essential amino acids and hydroxyproline and cystine each tested individually at 20mM concentrations with the PAT/phosphinothricin combination.

Since acetylation of L-glutamate has been reported in the literature to be a low affinity side reaction, this amino acid was also tested separately (at concentrations of 0, 0.0033, 0.033, 0.33, 3.3, 33 and 166mM) as a co-reactant with ^{14}C phosphinothricin (0.3mM).

To check the direct competition of glutamate with phosphinothricin, equimolar concentrations of ^{14}C phosphinothricin and 1- ^{14}C glutamate were incubated with sufficient PAT enzyme to convert all the phosphinothricin to acetyl-phosphinothricin in 0.1 minutes. The reaction were incubated and monitored for 90 minutes. Samples were removed at 5, 15, 30, 45, 60 and 90 minutes and run on TLC.

The final experiment was to determine if any other amino acid was acetylated using ^{14}C acetyl-CoA with the amino acid profile described above. Incubation times were not given.

Inactivation in Canine Gastric Fluid

Samples of fluid from beagle dogs was incubated with PAT at 37° to determine the rapidity of inactivation. The gastric fluids were also partially neutralized with NaOH to determine the effect of higher pH on the rate of inactivation. The PAT enzyme was studied as both the purified extract and a partial extract from plant material. The inactivation reaction was stopped by addition of the digests to a buffer (final pH=8). No details of this experiment were presented such as how the enzyme activity was determined or how the plant extracts were prepared.

Presence of PAT on Expression of Tomatine in Tomatoes

The possible pleiotropic effect of introducing the PAT gene into the levels of known inherent plant toxins was examined in wildtype

and transgenic tomato. The details of this analysis are not presented but a summary table presents levels detected.

RESULTS

The purification resulted in a single SDS-PAGE band with an apparent molecular weight of 22kD (attached photograph of gel is upside down). The results of the temperature and pH characterization indicate PAT activity above 20% of optimal from pH 6 to approximately 11 and between 35° and 65°C. The enzyme kinetics data indicate a $K_m(\text{acetyl-CoA})$ of 0.6mM and $K_m(\text{L-phosphinothricin})$ of 0.3mM. The substrate specificity data shows no acetylation of any of the tested amino acids by themselves or interference with the acetylation of phosphinothricin. Reported affinity between PAT and glutamate was not found in the range of 0.0033 mM to 166mM tested nor was any acetylation of glutamate detected after 90 minutes incubation.

The purified PAT enzyme was inactivated in under 1 minute in full strength canine gastric fluid (pH 1.1) and much less rapidly as the pH was raised above 2. The activity was reduced to about 50% after 8 minutes incubation once the pH was raised to 4. The results with a plant extract gave similar results at pH 1.1 but the rate of inactivation was slower with the higher pH's (approximately 10 minutes at pH 4 to reach 50% inactivation).

The data on tomatine levels that for plants derived from this particular transformation event, there does not appear to be any gross difference in the levels of this alkaloid between the wildtype and transgenic tomato expressing PAT.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD
Secondary Reviewer: Roy D. Sjoblad, Ph.D., Microbiologist, BPPD

STUDY TYPE: In vitro Digestibility
MRID NO: 437669-29
CHEMICAL NO: 128850 Glufosinate-ammonium
TEST MATERIAL: L-Phosphinothricin N-Acetyltransferase
STUDY NO: A51230
SPONSOR: AgrEvo USA Company, Wilmington, DE
TESTING FACILITY: Hoechst Schering, Frankfurt, Federal Republic of Germany
TITLE OF REPORT: L-Phosphinothricin N-Acetyltransferase In activation by Pig and Cattle Gastric Juice
AUTHOR: Dr. Arno Schulz
STUDY COMPLETED: August 31, 1993
CONCLUSION: The incubation of a crude plant extract with detectable PAT activity in porcine and bovine stomach fluids caused a rapid decrease in the detectable level of PAT activity. If the pH of these fluids was raised, the PAT activity did not decrease as rapidly.
CLASSIFICATION: Supplemental. Some details of the analysis were not provided such as source and method of obtaining the digestive fluids, PAT assay incubation times and determination of PAT specific activity. Based on other information presented in this package, this study does not need to be upgraded.

STUDY DESIGN

Inactivation of the PAT enzyme present in plant extracts was monitored in porcine and bovine gastric juices using ¹⁴C labelled L-phosphinothricin.

Test Method

A crude extract of an unidentified plant was prepared by grinding one part leaf tissue with 4 parts extraction buffer (25mM Tris/HCl; 5mM 2-mercaptoethanol; 1% polyvinylpyrrolidone, pH 7.5) at 4°C. This extract was filtered(?), centrifuged at 20,000 xg and the clear supernatant was used as an enzyme source. "An appropriate amount of enzyme extract" for the tests apparently related to the 0.78 pkat/mg protein specific PAT activity. PAT enzymatic activity was assayed by incubation with 2µl of 50mM acetyl-CoA, 5µl of ¹⁴C-phosphinothricin at 10 mM with 100,000 dpm and 8µl of buffer (25 mM Tris/HCl and 5 mM mercaptoethanol, pH 7.5). After an unspecified incubation time, the reaction was stopped with addition of 5µl of 2.5N H₂SO₄, centrifuged and the supernatant analyzed for acetylated phosphinothricin by separation on HPLC with an Aminex HPX 87H column with 0.01N H₂SO₄ as the running buffer. The radioactivity was detected with a Raytest ramona 90 HPLC counter.

The inactivation studies were carried out at 37°C with a 7:3 mix of stomach fluid to crude plant extract. The stomach pH was adjusted with the addition of NaOH. At the appropriate time intervals (0.5, 1.0, 2.5, 5.0, 10 & 15 minutes), 40µl samples were removed. This sample was immediately added to a vial with 20µl of ice-cold Tris/HCl at pH 8.2 to neutralize the reaction and 18µl of this extract were then analyzed for PAT activity as described in the paragraph above. Preparation of the porcine stomach and bovine "rennet-bag" and "paunch" fluids was not described.

RESULTS

PAT activity was reduced to approximately 20% by the first 30 second incubation in both porcine stomach and bovine rennet-bag fluid (pH 1.7 and 1.28, respectively). As these fluids were neutralized with NaOH up to pH 5.5 and 6.37, respectively, there was a longer duration of the PAT activity. After incubation for up to 15 minutes at these higher pH's, PAT enzyme activity was reduced in both porcine and "rennet-bag" fluid to 30% and 80% of full activity, respectively. In the "bovine paunch fluid" the activity did not decrease until after 5 minutes incubation.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD
Secondary Reviewer: Roy D. Sjoblad, Ph.D., Microbiologist, BPPD

STUDY TYPE: In vitro Digestion
MRID NO: 437669-30
CHEMICAL NO: 128850 Glufosinate-ammonium
TEST MATERIAL: L-Phosphinothricin N-Acetyltransferase
STUDY NO: A51613
SPONSOR: AgrEvo USA Company, Wilmington, DE
TESTING FACILITY: Hoechst Schering, Frankfurt am Main, Federal Republic of Germany
TITLE OF REPORT: Fate of Introduced DNA in Gut: Degradation of Phosphinothricin Acetyl Transferase Gene from Transgenic Rape HCN 92 (*Brassica napus*) in Stomach Fluids from Pig, Chicken and Cow
AUTHOR: Dr. R. Schneider
STUDY COMPLETED: November 11, 1993
CONCLUSION: The PAT gene was not detectable by PCR after an hour's incubation of HCN92 crude plant extracts incubated in normal gastric fluid from cow and chicken. Functional copies of the PAT gene were detectable by PCR in all HCN92 crude plant extracts treated with gastric fluids with increased pH. Purified DNA from HCN92 was not able to transform *Escherichia coli* cells before or after gastric fluid incubation.
CLASSIFICATION: Supplemental.

STUDY DESIGN

The integrity of DNA introduced into pig, chicken and cow gastric digestive fluids was examined by use of PCR and *E. coli* transformation.

Test material

Plant leaves from rape line HCN 92 (*Brassica napus*) expressing the PAT gene were taken from 6 week old greenhouse grown plants. Digestive fluids from the stomach of pig and chicken were obtained from Hoechst's Animal Health Group. Stomach fluids with normal pH or adjusted to higher pH's were used to incubate the plant tissue. PCR primers for the PAT gene were synthesized on an Applied Biosystem Type 391 DNA synthesizer. Reagents for the DNA reaction were from Applied Biosystems and MWG. Taq-Polymerase was from Perkin-Elmer. Restriction enzymes were from Boehringer-Mannheim, DuPont and Stratagene. The blot membranes and radioisotopes were from DuPont-NEN. The pOCA 18 vector containing a copy of the inserted PAT gene was provided by Dr. P. Eckes, Hoechst AG.

Test method

DNA Purification:

One half gram of fresh leaf tissue was crushed with 2.5 ml of the

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD
Secondary Reviewer: Roy D. Sjoblad, Ph.D., Microbiologist, BPPD

STUDY TYPE: Product Characterization
MRID NO: 437669-31
CHEMICAL NO: 128850 Glufosinate-ammonium
TEST MATERIAL: L-Phosphinothricin N-Acetyltransferase
STUDY NO: A53356
SPONSOR: AgrEvo USA Company, Wilmington, DE
TESTING FACILITY: Hoechst Schering
TITLE OF REPORT: Expression of the Phosphinothricin
Acetyltransferase in Glufosinate Resistant T14
and T25 Corn
AUTHOR: Dr. Arno Schulz
STUDY COMPLETED: December 1, 1994
CONCLUSION: PAT enzyme activity was found in crude
extracts of corn roots, leaves, stems and seed
for two different transformation events.
Pollen did not have detectable PAT activity.
CLASSIFICATION: Supplemental. No PAT values for control non-
transformed tissue extracts were presented.

STUDY DESIGN

Expression of the PAT gene in different tissues of transformed corn was examined using crude extracts and ¹⁴C labelled phosphinothricin substrate.

STUDY METHODS

Test Material Leaves, roots, stem, pollen and seed were collected from progeny of crosses with glufosinate resistant corn plants. These progeny were designated T14 (KW5361 x KW1292⁽⁴⁾T14) and T25 (LH82⁽⁴⁾ x T25sf⁽⁴⁾). The plants were greenhouse grown and tissues harvested when the plants began to flower. All tissues were collected, frozen in liquid nitrogen and stored at -80°C no longer than one week prior to extraction. Seeds from the original batch were stored at room temperature until extraction.

Extraction Method Seeds were crushed, extracted with ice-cold buffer (20mM phosphate, 1mM EDTA, 1mM DDT, pH 7.5) for 30 minutes and the resulting slurry centrifuged for 10 minutes at 15,000 xg. The other tissues were ground with a mortar and pestle with some sand and buffer. The resulting slurry was centrifuged for 10 minutes at 15,000 xg. The clear supernatants were used for the enzyme assays.

Enzyme Assay The PAT enzyme assay was described in MRID 437669-29 but additional details are provided here. The specific activity of the L-¹⁴C-phosphinothricin was stated as 1826 MBq/g or 361.9 MBq/mMole when used in the pollen and root tissue assays. This activity was diluted to 28.9 MBq/mMole with unlabelled phosphinothricin for assays with leaves and stem tissue. An assay

mixture consisting of 80 μ l phosphate buffer (20mM, pH 7.5), 20 μ l acetyl-CoA (50mM, pH 7.5) and 50 μ l 14 C-phosphinothricin (10mM) was prepared. 15 μ l of this mix added to 10 μ l of crude enzyme extract described above was incubated for 60 minutes at 37°C prior to being stopped with 5 μ l of 5N H₂SO₄: methanol (1:1). The mixture was centrifuged, followed by HPLC analysis. HPLC was with a 5 μ m Sephasorb SAX column (250 x 4.5mm) run with 5mM phosphate, pH 1.95: methanol (90:10) at a flow rate of 0.7ml/min. The radioactivity was detected with a Raytest RAMONA radiodetector.

RESULTS

PAT activity was not found in pollen samples but was detectable in all the other corn tissue samples examined the results are summarized below. The average specific activity in the stem and leaf tissues is higher than the other tissues. However, the T14 root tissue gave the overall highest PAT activity which speaks to the inherent variability due to different transformation events and this impact on the subsequent trait's expression. Another possibility for the high PAT activity in the root tissue is contamination by soil microbes. However, the level is fairly consistent (~70mU/mg) in three of the four samples reported so microbial contamination may be unlikely.

TISSUE TYPE/TRANSFORMANT	PAT (mUnits/mg protein)
ROOT/T25	5.4 \pm 4.7
ROOT/T14	91.1 \pm 31.6
LEAF/T25	41.3 \pm 6.5
LEAF/T14	23.8 \pm 6.8
STEM/T25	50.9 \pm 10.7
STEM/T14	38.1 \pm 14.6
POLLEN/T25	none detected
POLLEN/T14	none detected
SEED/T25	0.68 \pm 0.6
SEED/T14	3.9 \pm 0.8

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD *gjk*
Secondary Reviewer: Roy D. Sjoblad, Ph.D., Microbiologist, BPPD *RPS*

STUDY TYPE: Product Characterization
MRID NO: 437669-32
CHEMICAL NO: 128850 Glufosinate-ammonium
TEST MATERIAL: L-Phosphinothricin N-Acetyltransferase
STUDY NO: A53391
SPONSOR: AgrEvo USA Company, Wilmington, DE
TESTING FACILITY: Hoechst Schering
TITLE OF REPORT: Comparison of the Phosphinothricin
Acetyltransferase Enzyme Expressed in
Escherichia coli, Corn (T14 and T 25) and
Canola (HCN-92)
AUTHOR: A. Schulz
STUDY COMPLETED: December 1, 1994
CONCLUSION: PAT protein was detectable by western blot in
the root and leaf extract of two transformed
lines of corn (T14 & T25) and a line of canola
(HCN92). No PAT was visible in the wild-type
corn or canola.
CLASSIFICATION: Acceptable.

STUDY DESIGN

An immunological comparison of PAT enzyme as produced in two corn lines, canola and *E. coli* was made by western blot

STUDY METHODS

Plant Materials Leaves were taken from greenhouse grown plants beginning to flower and roots were from sterile two-week old seedlings germinated on filter paper. The sources were canola (*Brassica napus* HCN92), corn (*Zea mays* T14 & T25) and *Escherichia coli* J 118 Ac). The anti-PAT antibody was generated in a goat using purified PAT from the over-expressing *E. coli* strain.

Tissue Extraction Tissue was frozen in liquid nitrogen and ground in a mortar and pestle with extraction buffer (25mM Tris/HCl, 5mM 2-mercaptoethanol, 0.1mM phenylmethylsulfonylfluoride, pH 7.5, with 2-3 ml of buffer/g fresh weight tissue) and sand. After filtration through three layers of miracloth and centrifugation for 20 minutes at 15,000 xg, the crude extracts were used as enzyme sources. A Bio-Rad protein assay (Bradford method) was run on the extracts and they were apparently subsequently concentrated with a Centricon-10. The samples were then run on two separate 15% PAGE-SDS gels. One gel was stained directly for protein and the other blotted onto a nitrocellulose membrane for reaction with antibody and subsequent staining.

RESULTS

PAT protein itself was seen as comigrating bands in western blots of the crude extracts of root and leaf tissue from all the expected

positive samples tested. The extracts of non-transformed canola and corn showed no detectable bands at the location expected for the PAT protein in the western blots. In the lanes with purified PAT protein added to the tissues extracts, the intensity of the PAT positive band increased and no additional bands were seen. In the gels stained for total protein, no detectable band was seen in the lane with the 100ng of PAT protein purified from *E. coli*. In this same lane of the western blot a clear band was visible which comigrated with the antibody reactive bands in the other tissues extracts. No total protein stained gel was presented for the canola extracts.

BPPD COMMENTS

The comigrating bands for PAT protein and the lack of extra bands when purified extracts are added to the plant extracts are a convincing proof of their presence in tissues tested. The lack of a detectable band in the PAT protein in total protein stains of the gel is unfortunate as is the omission of the sizes for the molecular weight standards. It is interesting to note that this assay shows no detectable difference in the intensity of staining for the PAT protein although the PAT enzymatic activity assays indicated a gross difference in the level of activity especially in the root tissue of lines T14 and T25. This may speak to the problem of microbial contamination for the enzyme assays or may be due to the difference between seedling and mature root tissues used as samples in these assays.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD
Secondary Reviewer: Roy D. Sjoblad, Ph.D., Microbiologist, BPPD

STUDY TYPE: Product Characterization
MRID NO: 437669-33
CHEMICAL NO: 128850 Glufosinate-ammonium
TEST MATERIAL: L-Phosphinothricin N-Acetyltransferase
STUDY NO: A53504
SPONSOR: AgrEvo USA Company, Wilmington, DE
TESTING FACILITY: Hoechst Schering
TITLE OF REPORT: Comparison of the Synthetic PAT Gene and the PAT Protein with Other Known Nucleotide and Protein Sequences
AUTHOR: P. Eckes
STUDY COMPLETED: November 29, 1994
CONCLUSION: The DNA and amino acid sequence data indicate that the PAT protein is not related to proteins known to be toxic or allergenic to mammalian species. The PAT proteins appear to be most closely related to themselves and are not apparently closely related to other acetyl transferase enzymes.
CLASSIFICATION: Acceptable.

STUDY DESIGN

The nucleotide and amino acid sequence of the PAT gene and protein were compared with other known sequences in the EMBOL and SWISSPROT databases.

STUDY METHODS

The DNA and amino acid sequence of the synthetic gene generated for PAT expression in plants was compared to those found in the available databases.

RESULTS

The synthetic PAT gene shows significantly lower DNA homology (~70%) to its source of origin in *Streptomyces viridochromogenes* than might be expected. The fact that the original gene had a GC content of over 90% would probably make it unlikely to be efficiently expressed in plants since their DNA has a much lower GC content. Outside of the gene sequences for PAT from *S. viridochromogenes* and *S. hygroscopicus* and their synthetic derivatives, no significant areas of homology occurring across the entire gene were to be found in any other organisms.

The amino acid sequence comparison yielded similar results except that another source of the PAT gene was identified that was not found in the DNA database: *Alcaligenes faecalis*. In addition, there is a protein from *Streptomyces coelicolor* that has significant homology to a large portion of the PAT protein (32% identity over a 97 aa overlap). This protein is referred to as

"hypothetical protein in HRDD region (open reading frame X)." The other significant homology (25.8% in a 120 aa overlap) is with the human Ig heavy chain precursor V-II region (ARH-77). The other homology matches listed are for smaller regions of overlap.

BPPD COMMENTS

The DNA homology work, while showing how DNA with only 70% homology can still code for an identical protein, is not really relevant for addressing hazards or risks associated with this trait. The protein amino acid homology data will be useful for establishing what can be a cutoff for "similar proteins" and how amino acid similarity does not necessarily correlate with protein function. It should be noted that the lack of consensus sequences for glycosylation sites and the comigration of plant and microbially expressed proteins is fairly substantial support for lack of glycosylation in the absence of actual carbohydrate testing of the protein.

DATA EVALUATION REPORT

Reviewed by: John L. Kough, Ph.D., Biologist, BPPD
Secondary Reviewer: Roy D. Sjoblad, Ph.D., Microbiologist, BPPD

STUDY TYPE: In vitro Digestion
MRID NO: 437784-03
CHEMICAL NO: 128850 Glufosinate-ammonium
TEST MATERIAL: L-Phosphinothricin
STUDY NO: A53425
SPONSOR: AgrEvo USA Company, Wilmington, DE
TESTING FACILITY: Hoechst Schering AgrEvo Limited, Frankfurt am Main, Federal Republic of Germany
TITLE OF REPORT: Digestion of the Phosphinothricin Acetyltransferase Enzyme in Human Gastric Fluid (Simulated)
AUTHOR: A. Schulz
STUDY COMPLETED: December 1, 1994
CONCLUSION: PAT enzyme, either purified or in a plant extract, is rapidly degraded in gastric fluids as monitored by immunorecognition and enzyme activity. If pepsin is omitted from the gastric fluid, the PAT enzyme is fairly stable being recognized in a western blot after 180 minutes and is still enzymatically active (~10% full activity) after 120 minutes.
CLASSIFICATION: Acceptable.

STUDY DESIGN

The activity and immunorecognition of PAT protein, either purified or in crude plant extract, was examined after incubation in simulated human gastric fluid.

STUDY METHODS

Materials Gastric fluid was prepared as described in the U.S. Pharmacopeia using 2g of NaCl, 3.2g pepsin dissolved in 7.0 ml of HCl and sufficient water to yield 1000 ml. The pepsin was diluted to give a value between 1.0 and 1.7 units activity/mg. The crude plant extract was done with leaves from greenhouse grown, flowering corn (line T14) that were frozen and extracted as described in MRID 437669-32. Pat protein purified from an over-expressing *E. coli* was also used at a protein concentration of 150µg/ml.

Digestion and Activity Measurements The incubations were done at 37°C by addition of 20µl of the protein samples to 180µl of prewarmed gastric fluid. At the appropriate time, 20µl samples were removed from the incubation and placed in either 8.4µl of stop solution (66µl 20% SDS, 66µl 10x SDS-PAGE sample buffer, 33µl 2-mercaptoethanol and 18µl 1.8M NaOH) or on ice until the enzyme activity assay. For the SDS-PAGE the samples were boiled for 5 minutes at 100°C prior to loading onto the gel and running by standard protocols. The PAT activity assays were done with an assay mixture consisting of 80µl phosphate buffer (20mM, pH 7.5),

20 μ l acetyl-CoA (50mM, pH 7.5) and 50 μ l 14 C-phosphinothricin (10mM) was prepared. 15 μ l of this mix added to 10 μ l of crude enzyme extract described above was incubated for 60 minutes at 37°C prior to being stopped with 5 μ l of 5N H₂SO₄: methanol (1:1). The mixture was centrifuged, followed by HPLC analysis. HPLC was with a 5 μ m Sephasorb SAX column (250 x 4.5mm) run with 5mM phosphate, pH 1.95: methanol (90:10) at a flow rate of 0.7ml/min. The radioactivity was detected with a Raytest RAMONA radiodetector.

RESULTS

PAT protein, either purified or in a tissue extract, is rapidly degraded in gastric fluid according to the results from both western blots and enzyme activity assays. The presence of plant extract slightly slows the degradation as tracked by western blot since a faint immunoreactive band that comigrates with the PAT band and a lower molecular weight band are still visible after 5 seconds incubation. In the purified protein preparation, no PAT is visible at any incubation time point save the initial 0 minute time. If pepsin is omitted from the reaction mixture, essentially a low pH incubation, no significant degradation is visible over the 180 minute incubation.

The detectable enzyme activity in digested plant extracts decreases to less than 20% after one minute but does not approach 0 until nearly three minutes incubation. If pepsin is omitted from the reaction mixture, the enzyme activity in the plant extract decreases to about 20% after 2 minutes but remains consistently at about 10% of full activity even at 120 minutes incubation.



13544



R136712

Chemical: Glufosinate

PC Code:
128850

HED File Code: 41500 BPPD Tox/Chem

Memo Date: 1/16/2006

File ID: DPD219909

Accession #: 000-00-9001

HED Records Reference Center
2/1/2007

